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Supplemental Information

Protective plant immune responses are elicited by bacterial outer membrane vesicles

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Supplementary Figure 1

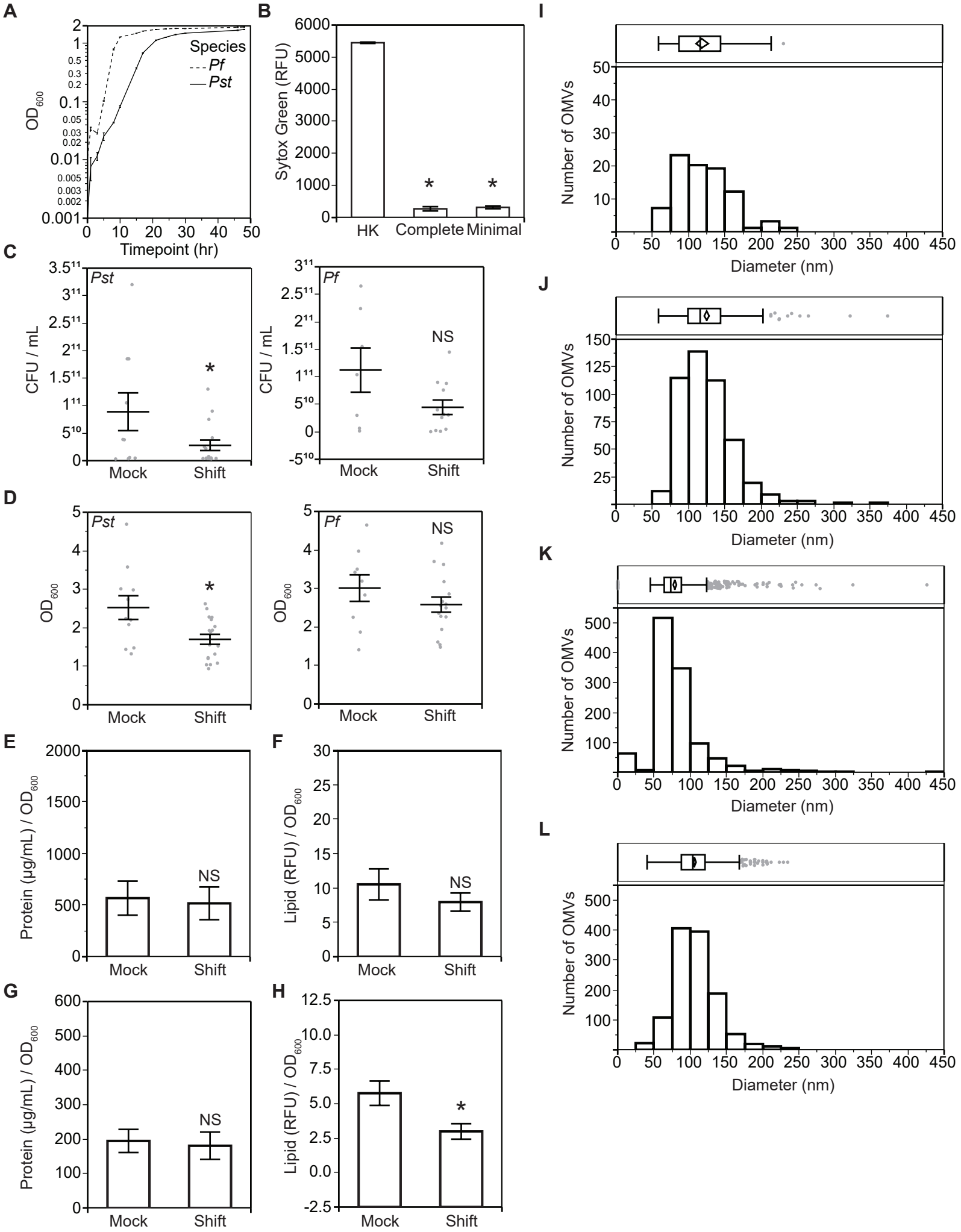


Figure S1. *Pseudomonas syringae* and *Pseudomonas fluorescens* outer membrane vesicles (OMVs). Related to Figure 1.

A. Growth of *Pst* and *Pf* measured by OD_{600} in 1L complete media cultures. n=3. **B.** Membrane integrity of *Pst* in complete versus minimal media at time of OMV harvest as measured by Sytox Green where fluorescence intensity indicates compromised membrane integrity. HK: heat-killed bacterial cells. Statistics: ANOVA, Tukey HSD. n=3. **C.** CFU/mL of *Pst* (left) and *Pf* (right) in complete versus minimal media. Complete media cultures were mock-shifted to complete media (Mock) or shifted to minimal media (Shift) for 2 h. Statistics: Two-tailed Student's T-test. *Pst* Mock: n=10; *Pst* Shift: n=15; *Pf* Mock: n=7; *Pf* Shift: n=11. **D.** OD_{600} of *Pst* (left) and *Pf* (right) grown in complete media and mock-shifted to complete media (Mock) or shifted to minimal media (Shift) for 2 h. Statistics: Two-tailed Student's t-test. *Pst* Mock: n=12; *Pst* Shift: n=18; *Pf* Mock: n=9; *Pf* Shift: n=16. **E-H.** *Pst* (E, F) or *Pf* (G, H) OMV production as measured by (E, G) protein and (F, H) lipid normalized to culture density. Statistics: Two-tailed Student's T-test. Additional tests using repeated measures ANOVA, where CFU and OD_{600}, or protein and lipid were the repeated measures revealed decreased cell growth in minimal media and no difference in vesicle production, respectively. n≥3. **I-L.** Size distribution of OMVs isolated from *Pst* (I, K) or *Pf* (J, L) cultures grown in complete media (I, J) or grown in complete media and shifted to minimal media for 2hr (K, L). The box plot in the top panel summarizes the data where the vertical line is the median, left and right edges of the box are the 1st and 3rd quartile, respectively, left and right whiskers show the 1st quartile minus the interquartile range and the 3rd quartile plus the interquartile range, respectively, confidence diamond shows the mean, where the left and right edges of the diamond are the lower and upper 95% of the mean, respectively, and the points show outliers. *Pst* Complete: n=86; *Pf* Complete: n=466; *Pst* Shift: n=1104; *Pf* Shift: n=1448. Horizontal lines and error bars indicate mean ± SE. $p<0.05$ in all statistical tests. Asterisks indicate statistical significance. NS indicates not statistically significantly different.

Supplementary Figure 2

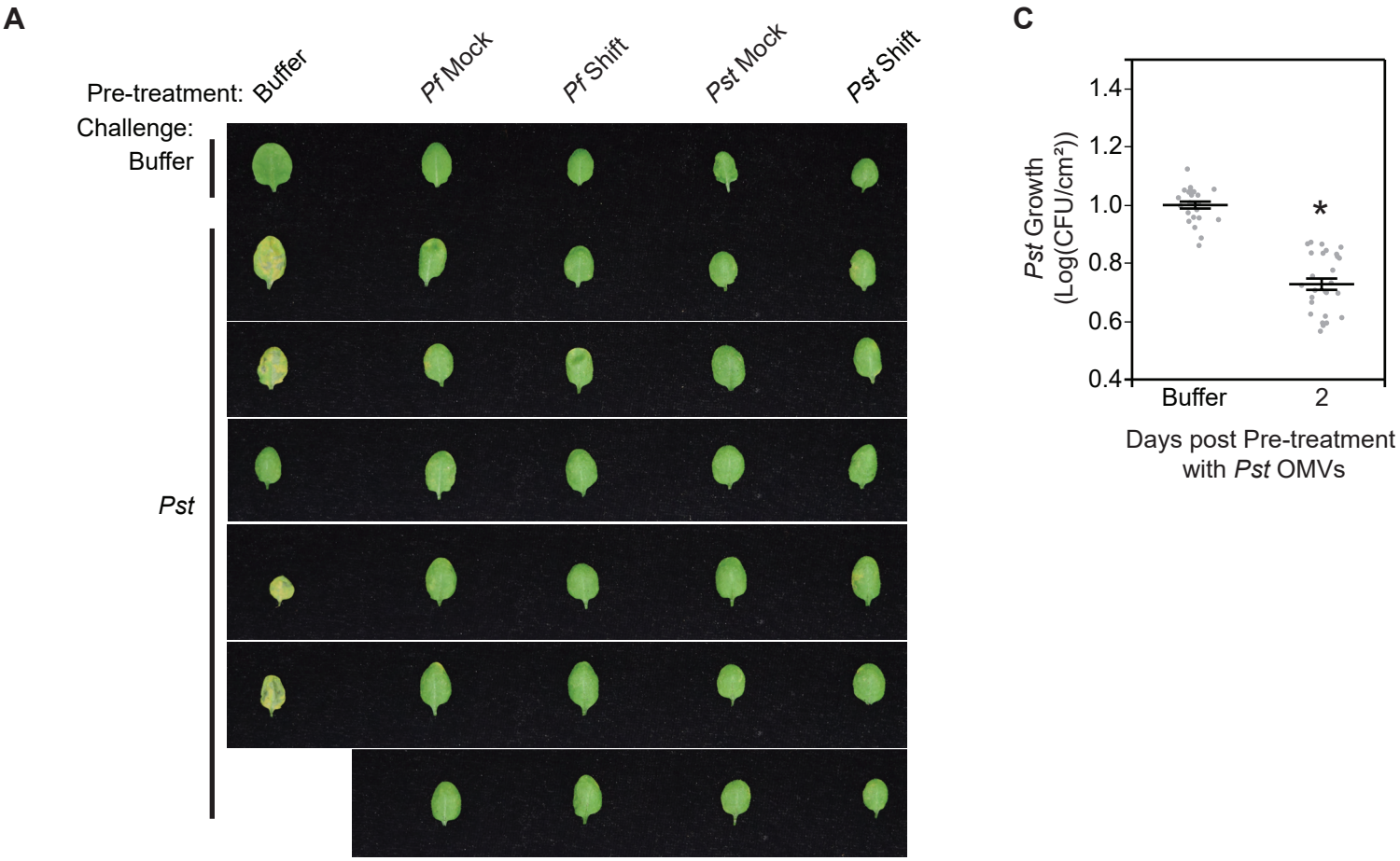


Figure S2. OMV treatment does not lead to *A. thaliana* leaf yellowing and protection lasts at least two days post infiltration. Related to Figure 1.

A. Additional images of protection against *Pst* challenge in *A. thaliana* leaves. Leaves were pre-treated with 5X *Pst* or *Pf* OMVs isolated from either complete (Mock) or minimal (Shift) media. **B.** Neither 5X *Pst* or *Pf* OMVs inhibit *Pst* growth. 5X *Pst* (Left) or *Pf* (Right) OMVs were added to cultures of *Pst* in complete media at the time of inoculation. OD_{600} was measured every 15 min for 48 h and compared to untreated cultures (No Treatment). Error bars are standard error. Statistics: Repeated Measures ANOVA. n=3. **C.** *Pst* in plants pre-treated with 5X *Pst* OMVs from minimal media cultures. Statistics: Two-tailed Student's T-test. n=3 experimental replicates, each with at least 7 plants per treatment condition. Gray scatter points display the value from each plant tested. Horizontal lines and error bars indicate mean ± SE. $p<0.05$. Asterisks indicates statistical significance.

Supplementary Figure 3

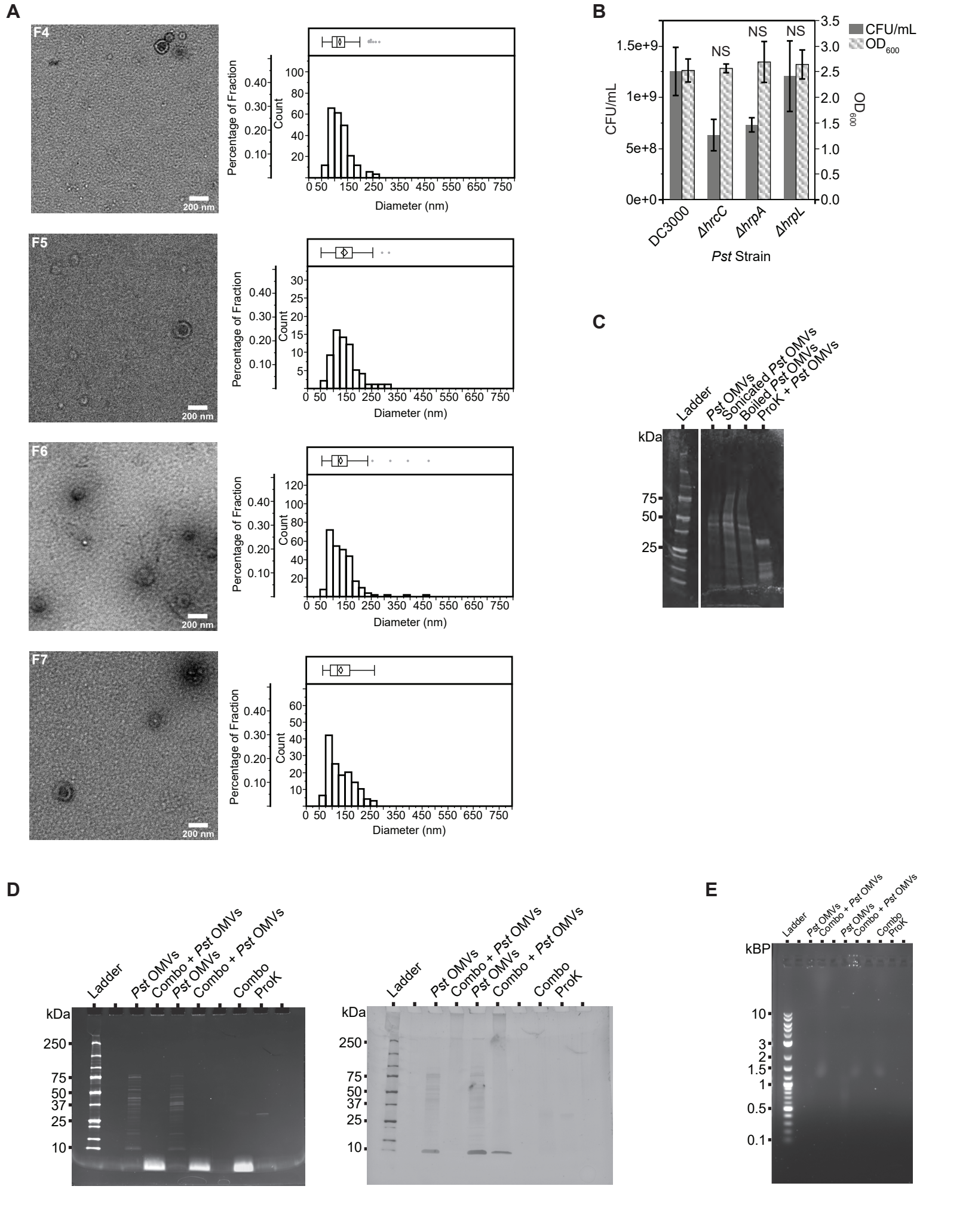
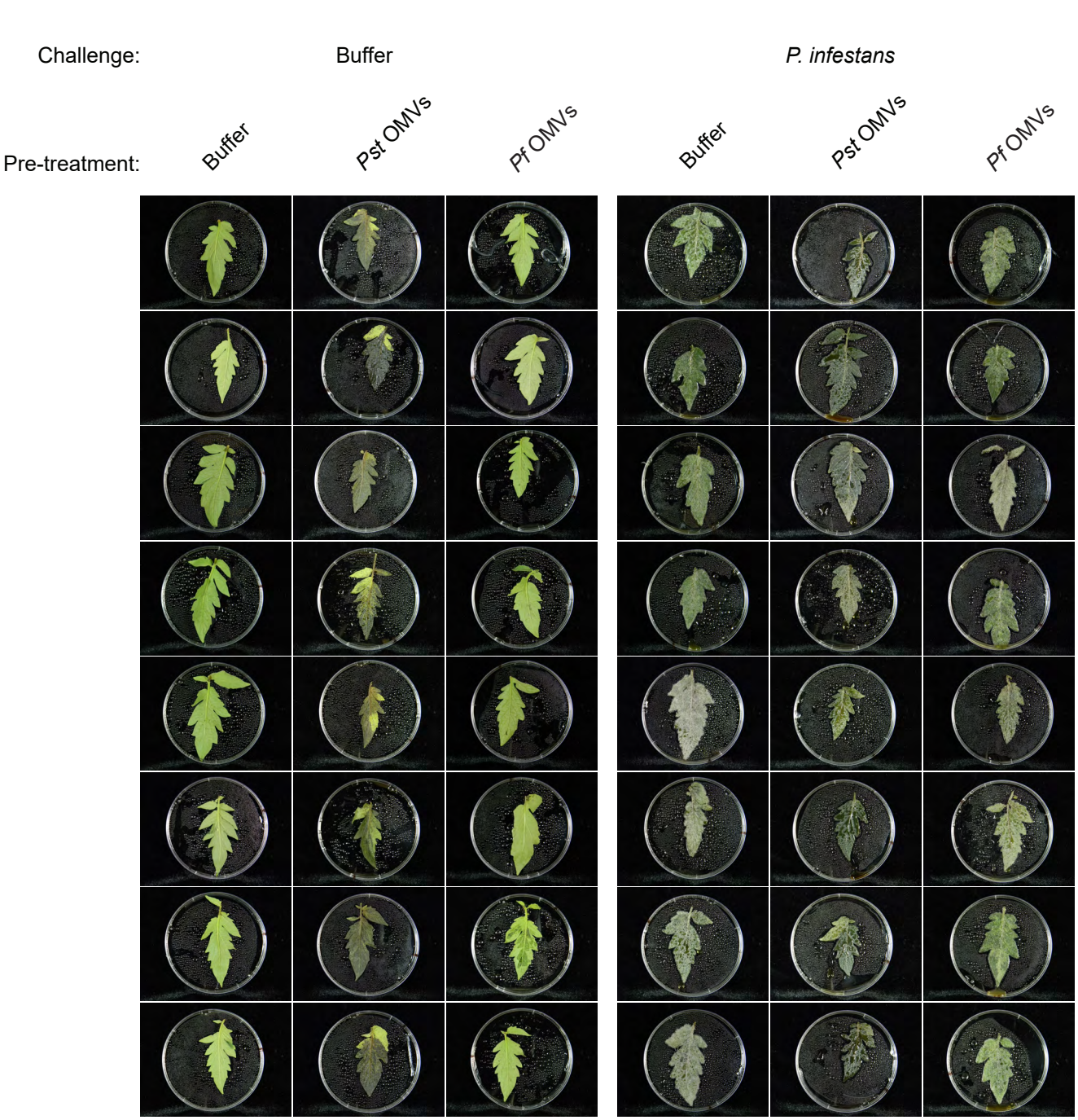


Figure S3. OMVs are present in fractions 4-7 of a density gradient, T3SS mutants display no growth defect *in vitro*, and treated vesicles do not contain detectable protein or nucleic acid. Related to Figure 2.

A. TEM images of *Pst* OMVs from OMV-containing fractions of the OptiPrep density gradient with the corresponding size distribution for each fraction. The density gradient was run using OMVs isolated from *Pst* cultures shifted to minimal media. For the size distributions, the box plots in the top panels summarize the data where the vertical line is the median, left and right edges of the box are the 1st and 3rd quartile, respectively, left and right whiskers show the 1st quartile minus the interquartile range and the 3rd quartile plus the interquartile range, respectively, confidence diamond shows the mean, where the left and right edges of the diamond are the lower and upper 95% of the mean, respectively, and the points show outliers. Scale bars: 200 nm. F4: n=225; F5: n=66; F6: n=257; F7: n=142. **B.** CFU / mL (left axis, solid bars) and OD_{600} (right axis, hatched bars) for *Pst* WT and T3SS mutants grown in complete media. Horizontal lines and error bars indicate mean ± SE. Statistics: Repeated Measures ANOVA using CFU and OD_{600} as repeated measures for growth. $p<0.05$. NS indicates not statistically significantly different. n=3. **C, D.** 5X OMVs from *Pst* cultures shifted to minimal media were untreated or treated with sonication, boiling, or Proteinase K (C) or combined (Combo) treatment (D) and separated by SDS-PAGE to examine protein contents using SYPRO Ruby Stain (C and D (Left)) and Silver Stain (D (Right)). Proteinase K is 28.9 kDa. **E.** 5X OMVs from *Pst* cultures shifted to minimal media were treated with combined treatment and run on a 1% agarose gel. In D and E, lanes 3-6 and 3-7, respectively, are replicates from two independent OMV preparations and corresponding treatments. In all gels, equivalent amounts of OMVs were added at the 5X concentration. ProK: Proteinase K. Vertical dashes without labels indicate blank lanes.

Supplementary Figure 4

A



B

Figure S4. *Pst* OMVs, but not *Pf* OMVs, induce water soaking in tomato, and *Pst* and *Pf* OMVs reduce *P. infestans* growth. Related to Figure 3.

A. Additional images of tomato leaves infiltrated with buffer, or 10X *Pst* OMVs or *Pf* OMVs isolated from minimal media cultures Leaves were then challenged with buffer (left 3 columns) as a negative control or *Phytophthora infestans* (right 3 columns) and imaged. **B.** Images of tomato leaves immediately following 10X OMV infiltration from (A). Representative images are shown from 1 of n=3 experimental replicates.

Supplementary Figure 5

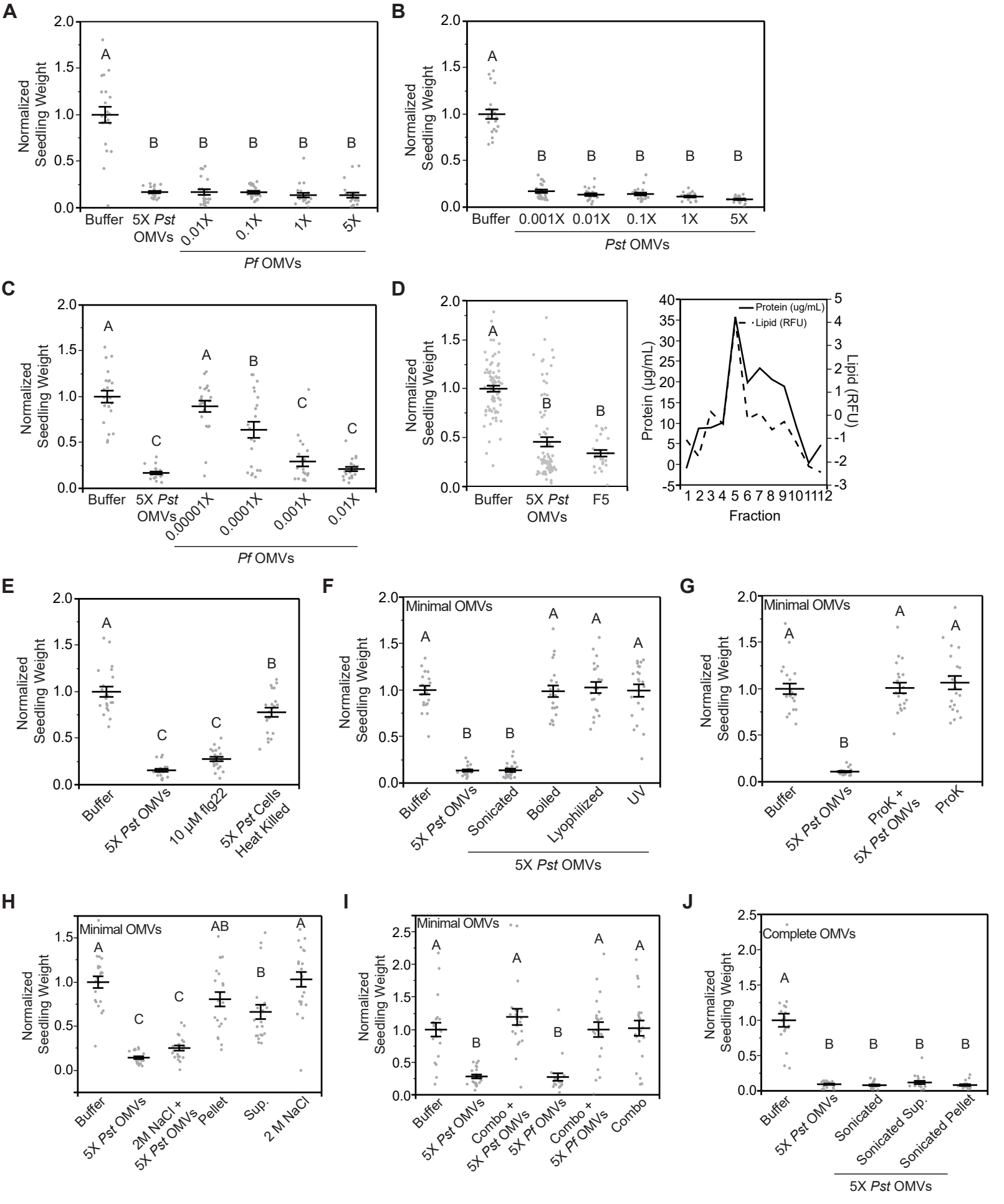


Figure S5. Different biochemical treatments have various effects on the growth inhibition activity of *Pst* and *Pf* OMVs from complete and minimal media. Related to Figure 4 and Table 1.

A-C. Seedling weight 7 days post treatment with either buffer or various concentrations of *Pf* OMVs from minimal media (A), *Pst* OMVs from complete media (B), or *Pf* OMVs from complete media (C). Statistics: ANOVA, Tukey HSD. **D.** (Left) Seedling weight 7 days post treatment with either buffer, 5X *Pst* OMVs from minimal media, or 5X *Pst* OMVs from fraction 5 of an OptiPrep density gradient. (Right) Protein and lipid concentration from each fraction of the OptiPrep density gradient used in (Left) with *Pst* OMVs from minimal media. Statistics: ANOVA, Tukey HSD. **E.** Seedling weight 7 days post treatment with either buffer, 5X *Pst* OMVs from minimal media, flg22, or heat killed *Pst* cells. Statistics: ANOVA, Tukey HSD. **F-J.** Data in Table 1: Seedling weight 7 days post treatment with OMVs from *Pst* or *Pf* from complete or minimal media as indicated at the bottom or in the top left of each graph, respectively. Treatments are indicated on the x-axis of each graph. Statistics: ANOVA, Tukey HSD. In all panels, $n \geq 3$ experimental replicates, each with at least 7 plants per treatment condition. Gray scatter points display the value from each plant tested. Horizontal lines and error bars indicate mean ± SE. $p<0.05$ in all statistical tests. Conditions not connected by the same letter are statistically significantly different.

Supplementary Figure 6

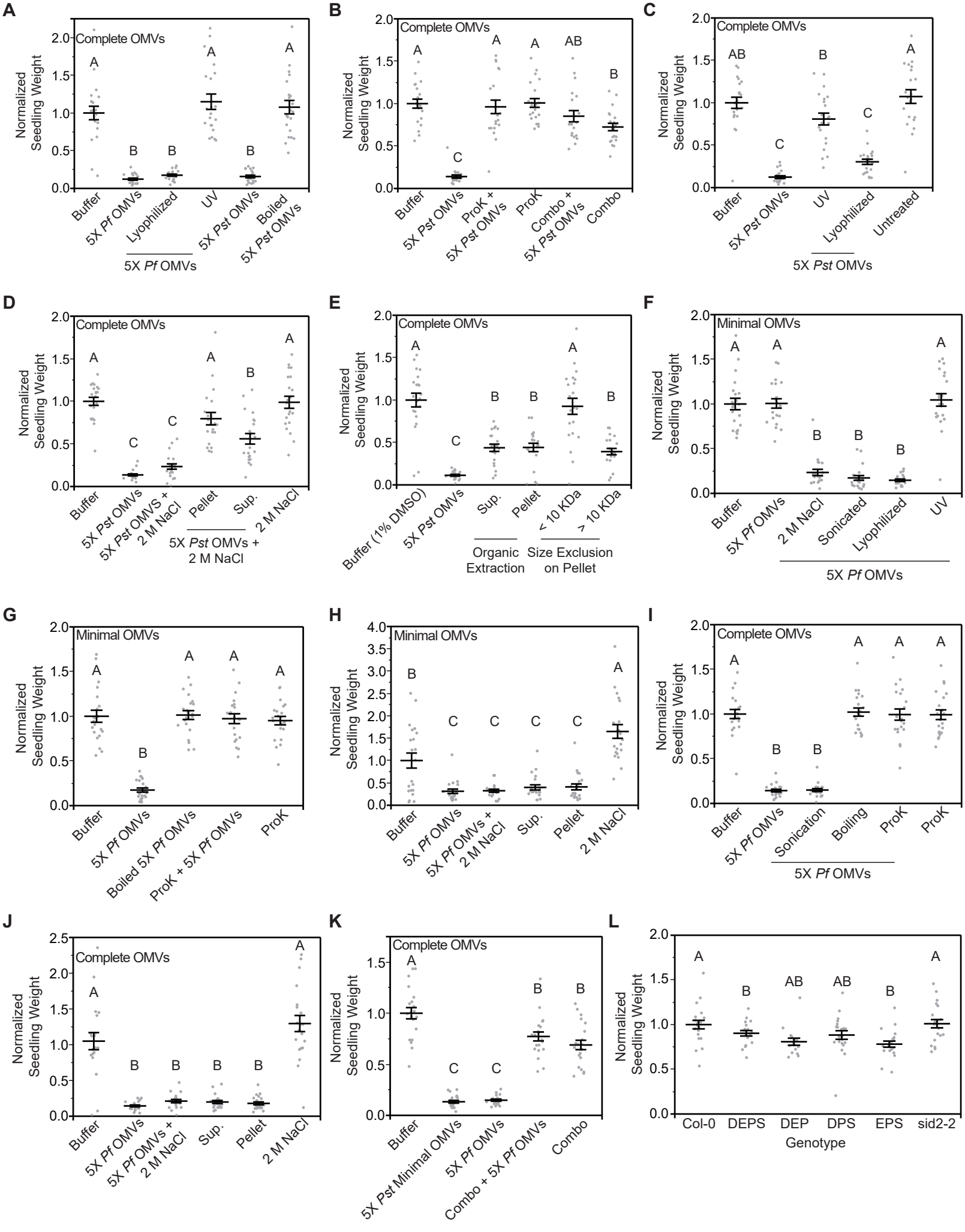


Figure S6. Different biochemical treatments have various effects on the growth inhibition activity of *Pst* and *Pf* OMVs from complete and minimal media (cont.). Related to Table 1.

A-K. Data in Table 1: Seedling weight 7 days post treatment with OMVs from *Pst* or *Pf* from complete or minimal media as indicated at the bottom or in the top left of each graph, respectively. Treatments are indicated on the x-axis of each graph. (E) Pst OMVs from complete media were divided into hydrophilic (Pellet) and hydrophobic (Sup.) fractions. The hydrophilic fraction was further divided into fractions containing particles less than or greater than 10kDa. Statistics: ANOVA, Tukey HSD. **L.** Seedling weight of untreated WT or mutant *A. thaliana* plants 14 days post germination. DEPS: *dde2-2/ein2-1/pad4-1/sid2-2*; DEP: *dde2-2/ein2-1/pad4-1*; DPS: *dde2-2/pad4-1/sid2-2*; EPS: *ein2-1/pad4-1/sid2-2*. Statistics: ANOVA, Tukey HSD. In all panels, $n \geq 3$ experimental replicates, each with at least 7 plants per treatment condition. Gray scatter points display the value from each plant tested. Horizontal lines and error bars indicate mean ± SE. $p<0.05$ in all statistical tests. Conditions not connected by the same letter are statistically significantly different.

Supplementary Figure 7

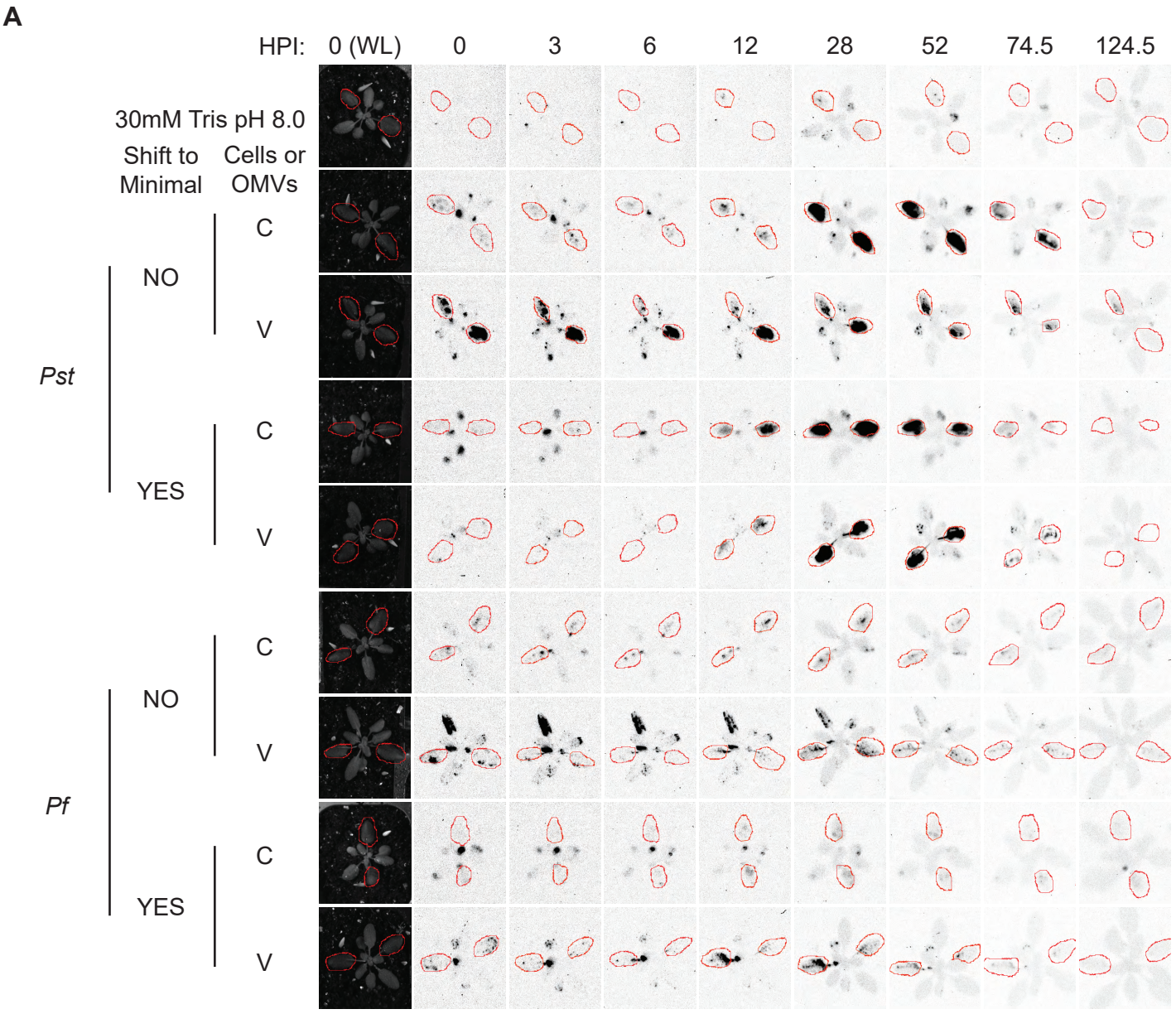


Figure S7. *Pst* OMVs induce ICS1 expression and SA/SAG accumulation. Related to Figure 5.
A. *ICS1* expression over time as shown by luminescence from Col-0 *ICS1*:LUC transgenic plants infiltrated with OD_{600} 0.002 cells (C) or 5X OMVs (V) from complete (NO) or minimal (YES) media. Red circles indicate infiltrated leaves. Dark shading indicates luminescence. WL: white light image. Representative images are shown from 1 of n=3 experimental replicates. **B.** Representative images of HPLC peaks from the SA/SAG purification. Slanted text displays area under the curve for the SA/SAG peak. Representative images are shown from 3 of n≥15.

Supplementary Figure 8

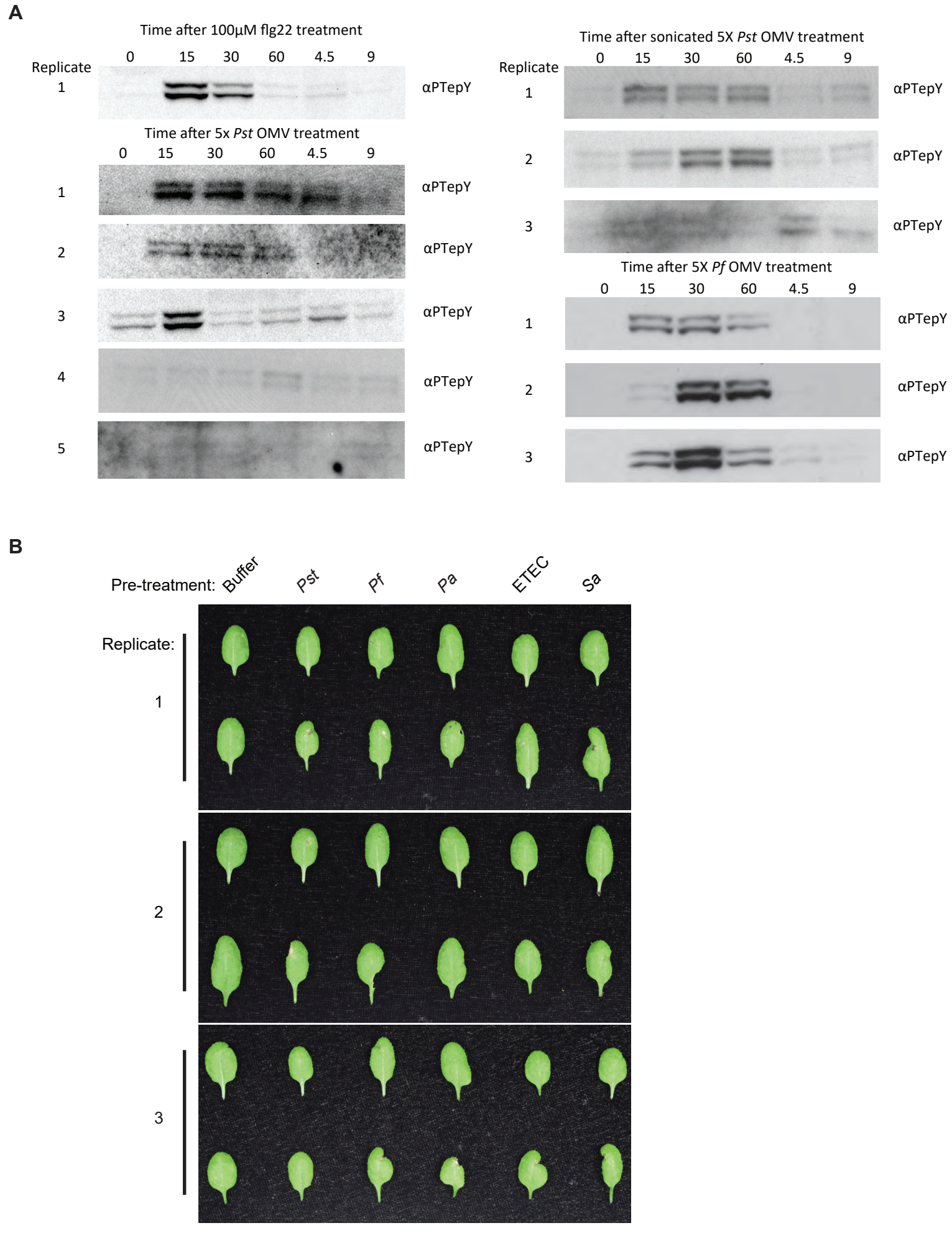


Figure S8. *Pst* OMVs induce MAPK activation and no OMVs/MVs tested result in leaf yellowing. Related to Figure 5.

A. 7-day-old seedlings were treated with flg22, 5X *Pst* OMVs, 5X sonicated *Pst* OMVs, or 5X *Pf* OMVs for the time indicated at the top of each panel. Western blots show phosphorylated MAPK for each replicate. **B.** Additional images of *A. thaliana* leaves showing no phenotype in response to treatment with OMVs/MVs from various species. Leaves were treated with 5X OMVs/MVs and imaged after 1 week in 16hr light/8hr dark conditions.

Supplemental Tables

Table S1. OMV Yield. Related to Figure 1.

Species	Media[a]	N	Culture Volume (mL)	CFU/mL	OD_{600}	Protein (µg/mL)	Lipid (RFU/100µL)
Pst	Complete-Complete	11	500	$9^{10} \pm 3^{10}$	2.5 ± 0.3	1224.8 ± 297.7	28.3 ± 9.6
Pst	Complete-Minimal	18	500	$3^{10} \pm 9^{9}$	1.7 ± 0.1	773.1 ± 218.9	13.3 ± 2.3
Pf	Complete-Complete	9	500	$1^{11} \pm 4^{10}$	3.0 ± 0.3	2250.8 ± 436.7	67.8 ± 10.0
Pf	Complete-Minimal	16	500	$4^{10} \pm 1^{10}$	2.6 ± 0.2	1727.1 ± 349.4	33.4 ± 8.6

[a]Yield from *Pst* and *Pf* cultures grown in complete media to early stationary phase and shifted to either complete or minimal media for 2 hr.